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A COMBINED TISSUE STAIN FOR THE SELECTIVE STAINING OF COLLAGEN, ELASTIC FIBERS AND ACIDIC CARBOHYDRATES*

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The histopathologic diagnosis of many skin diseases depends on the visualization of connective tissue components in the skin biopsy specimen. Collagen fibers, elastic fibers, and acid mucopolysaccharides form the three major extracellular connective tissue components of the dermis. A number of techniques are available for staining each of these tissue components separately. To study their relationship, however, it is useful to visualize all three in the same histologic section. Although in the past elastic tissue stains have been combined with a collagen stain or a stain for acidic carbohydrates (1), a technique for staining all three connective tissue components in the same histologic section combining highly selective stains for each component has not been available.

The present communication describes a relatively simple and reproducible technique for combining highly selective stains for collagen, elastic fibers and acidic carbohydrates for the histologic study of dermal lesions.

MATERIALS AND METHODS

Tissues Used

For the initial portion of the study while the technique was being developed, normal skin was obtained from the abdominal incision of 10 patients at autopsy. The tissue was fixed with neutral buffered formalin (1) and processed in a routine manner. Sets of 10 slides each were used for the various staining trials.

The evaluation of the technique is also based on the staining of a section from each skin lesion submitted to the surgical pathology laboratory for diagnostic and routine purposes over the period of approximately 18 months. During this time minor variations of dye concentration and staining time were tried for optimal results.

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Dyes

1. Acid fuchsin (for mixed collagen stain only)
2. Basic fuchsin
3. Hematoxylin
4. Alcian blue 8GX (George Gurr)
5. Sirius red F3BA (purchased from Verona Dyestuffs; P. O. Box 385; Springfield Road; Union, New Jersey).

When possible, all dyes used in histologic procedures should be certified by the Biological Stain Commission (2). Alcian blue 8GX and sirius red F3BA are commercial dyes and are not certified.

Chemicals

1. 3% acetic acid
2. 0.25% and concentrated hydrochloric acid (HCl)
3. Saturated aqueous picric acid. The solution should be prepared at least several hours prior to use and must have some undissolved crystals present.
4. 29% aqueous ferric chloride U.S.P.
5. Paraldehyde

Dye Mixtures

1. Alcian Blue (3)
   Add one crystal of thymol to prevent fungus growth. The solution keeps at room temperature indefinitely.
2. Gomori's Aldehyde Fuchsin (4)

Concentrated HCl	2 ml.
Paraldehyde	2 ml.
0.5% basic fuchsin in 70% ethanol	200 ml.

   Let the solution stand at room temperature for at least 24 hours. When it turns deep purple it is ready for use and must be stored in the refrigerator. Filter before each use. Staining becomes pale with extensive use or storage for some months.
3. Weigert's Iron Hematoxylin (5)

Stock Solution A	
Hematoxylin	1 gm.
95% ethanol	100 ml.
Stock Solution B	
Aqueous ferric chloride (29%)	4 ml.
Distilled water	95 ml.
Concentrated HCl	1 ml.
Working Solution	
Equal parts of A and B	

   The stock solutions keep indefinitely but the working solution deteriorates after 10–14 days.

4. Collagen Stain
   A. *Picrosirius red F3BA (6)*

Saturated aqueous picric acid	200 ml.
Sirius red F3BA	0.2 gm.

   B. *Mixed Collagen Stain*

Saturated aqueous picric acid	200 ml.
Acid fuchsin (pure dye)	0.03 gm.[1]
Sirius red F3BA	0.05 gm.
Concentrated HCl	0.5 ml.

   Both solutions A and B keep at room temperature indefinitely or until exhausted by extensive use.

Staining Procedure

1. Deparaffinize the tissue sections and hydrate them in running tap water (RTW) for 10 minutes.
2. Rinse in 3% acetic acid by dipping the staining basket into the solution ten times.
3. Stain in Alcian blue for 30 minutes.
4. Rinse in RTW for 3 minutes.
5. Rinse in 70% alcohol by dipping the staining basket into it ten times.
6. Stain in aldehyde fuchsin for 30 minutes.
7. Rinse in 3 changes of 70% alcohol by dipping the staining basket 10 times into each.
8. Rinse in RTW for 5 minutes.
9. Stain in working solution of Weigert's hematoxylin for 10 minutes.
10. Rinse in RTW for 10 minutes.
11. Stain in collagen stain A or B for 30 minutes.

11a. If B (Combined collagen stain) is used, rinse in 0.25% HCl by dipping the staining basket into it 5 times.

12. Dehydrate quickly in 3 changes of absolute ethanol by dipping the basket into each 10 times.
13. Clear in toluene or xylene and mount.

RESULTS

Nuclei are light brown and cytoplasm light yellow. Collagen stains red when stained with either one of the collagen stains. When picrosirius red F3BA is used alone, the large collagen bundles are often incompletely stained as has been discussed previously (7). The fine collagen fibers, particularly those adjacent to epithelial structures, stain a brilliant red. Occasionally, keratin of the stratum corneum stains red in some areas. On polarization microscopy, however, collagen is brilliantly birefringent, whereas no other substance stained with the picrosirius red becomes birefringent.

When the combined connective tissue stain containing sirius red and acid fuchsin is used, all the collagen is completely stained. The smaller fibers stain somewhat more with sirius red and appear brighter red than the larger fibers which stain more with acid fuchsin and stain a somewhat deeper red.

Acidic carbohydrates stain blue. These include polyanion substances containing carboxyl groups and/or sulfate groups and sialic acid-containing substances (so-called epithelial mucins). Aldehyde fuchsin, which is used to stain the elastic fibers, also stains some of the acidic substances. Since prior Alcian blue staining blocks most of the acidic binding sites, aldehyde fuchsin stains very little in the dermis other than elastic fibers. Some mast cell granules, sialomucin granules in eccrine sweat coils and fungi were found to have been stained by aldehyde fuchsin more strongly than with Alcian blue. Elastic fibers and internal elastic laminae of arteries, however, stain a brilliant purple with this stain.

DISCUSSION

Although the individual components of the technique presented here are well known and tried methods, their successful combination in the same histological section presents a distinct advantage.

In a study of various collagen staining techniques, Constantine and Mowry (8) found that none was specific for collagen. They found the picric acid methods to be the most selective, particularly when acid fuchsin or sirius red F3BA were used (7). They noted that while sirius red in the picrosirius red stained the finer collagen fibers more completely, the acid fuchsin in the picrofuchsin stained the larger collagen fibers better. Hence, it was reasoned here that a combination of the two dyes would give optimal results for staining both large and fine dermal collagen fibers. This, indeed, is the case. The combined picric acid sirius red-acid fuchsin stain is the best collagen stain in the author's experience. The only problem is the same as is true of acid fuchsin in general. Namely, that within a year or so certain batches of acid fuchsin have a tendency to fade. In those sections the collagen becomes partly decolorized. This is a distinct disadvantage if slides are to be kept permanently. However, if slides are prepared for temporary use and for

[1] To calculate the actual weight of acid fuchsin, divide 0.03 gm by the per cent of purity indicated on the container.

photography the combined connective tissue stain is recommended over the picrosirius red F3BA.

The concentrations of sirius red and acid fuchsin may be increased or decreased. Such changes, unless excessive, will affect the intensity of staining but not the selectivity. The length of staining may also be varied. Quite good results are obtained by staining for only 10 minutes, but 30 minutes seems optimal.

Of the standard techniques for staining elastic fibers, Gomori's aldehyde fuchsin is preferred. It shows good contrast with the collagen which is stained red. Aldehyde fuchsin does not stain collagen which has been altered by various chemical and physical procedures, whereas the other elastic tissue stains do (9). The Verhoff stain for elastic fibers often has a large degree of non-specific staining of nuclei, stratum corneum, and other structures unless the decolorization process is carried out extensively in which case many of the smaller elastic fibers tend to lose their staining. Sweat, *et al.* (6) have found that resorcin fuchsin is incompatible with sirius red F3BA. The fact that aldehyde fuchsin also stains some acidic carbohydrates poses no problem. Previous staining of the sections with Alcian blue blocks almost all of the acidic binding sites. For example, the mucin in adenoid basal cell carcinomas which has been described to stain with aldehyde fuchsin (10), stained with the Alcian blue only.

Acid orcein, initially introduced by Unna and popularized by Pinkus in his orcein-Giemsa stain (11), is also an excellent elastic tissue stain. Although it consistently stains nuclear material, the acid orcein stain is otherwise highly selective for elastic tissue. Those who consider it the best elastic tissue stain may substitute it (using certified synthetic orcein) for the aldehyde fuchsin stain. Steps 1 through 5 are performed as directed. Then the slides are stained in 1% orcein and 0.6% HCl in 70% alcohol for 45 minutes (30–60 minutes). A two minute rinse in distilled water, 10 dips in 95% alcohol, and 5 minutes in absolute alcohol follow. Then the slides are hydrated and washed in running tap water for 3 minutes. Following this the connective tissue procedure is resumed starting with step 11. Staining with hematoxylin is omitted since orcein stains the nuclei. The results are comparable with the procedure using aldehyde fuchsin except that the brown elastic fibers do not contrast as well with the red collagen.

The acidic carbohydrates are stained with Mowry's Alcian blue technique (3) with the exception that the staining time has been reduced from 2 hours to 30 minutes. Although the author agrees with Mowry that 2 hours of staining is optimal, the difference in intensity of staining between specimens stained 30 minutes and 2 hours is quite small. The 30 minute staining time is preferred since for this combined stain the 2 hours staining time is excessively lengthy. However, for those who prefer it, the 2 hour staining time may be used exactly as prescribed by Mowry (3).

Weigert's hematoxylin was found to produce good results with the technique. Harris' hematoxylin, which was initially tried, resulted in a reddish color of the nuclei as a result of the acid pH of the collagen-staining mixture.

The order of the staining procedure is important. Both Alcian blue and aldehyde fuchsin are extremely fast stains and either could be used first. Prior Alcian blue staining, however, renders aldehyde fuchsin more highly selective. The staining of elastic fibers is not affected by the Alcian blue presumably because elastic tissue staining is on the basis of hydrogen bonding (12). This is in contrast to staining of acidic carbohydrates, nuclei and collagen, all of which are primarily on the basis of ionic binding. Alcian blue is a basic dye which combines with the acidic groups of carbohydrates with great tenacity. Whereas many other basic dyes (*e.g.*, hematoxylin, Toluidine, blue, etc.) can be removed by exposing the section to a 1% HCl solution for a few seconds, Alcian blue is not affected. In Mowry's modification of the low pH Alcian blue staining technique for sulfated carbohydrates, the stained sections are exposed to 0.5 N HCl (about 1.8%) for a total of 9 minutes with no appreciable loss of color from the sulfated material (13).

Hematoxylin is a cationic dye which is not very fast; hence, it is removed by acidic solutions. The collagen staining mixture is acidic; therefore, it removes part of the hematoxylin. For this reason the slides are first overstained with hematoxylin prior to collagen staining. The picric acid method of collagen staining

must be the final step prior to dehydration since immersion in either water or alcohol for more than a few seconds will remove the picric acid and part of the sirius red and acid fuchsin.

This staining procedure was developed using formalin-fixed human tissues. Animal tissue or human tissues fixed with other fixatives may not yield similar results and must be evaluated individually.

SUMMARY

A relatively simple and reproducible histological procedure is described for the selective staining of three main extracellular connective tissue components in the same histological section. Acidic carbohydrates (both connective tissue and epithelial mucins) stain blue with Alcian blue, elastic fibers stain purple with aldehyde fuchsin and collagen stains red with sirius red F3BA with or without the addition of acid fuchsin. Mast cells and fungi are prominently stained with Alcian blue with some additional staining from aldehyde fuchsin. This procedure is recommended for the histological study of connective tissues in formalin-fixed human tissues, particularly skin lesions.

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